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Expression of polymorphic profiles of specific and nonspecific esterase in multivoltine and bivoltine silkworm, *Bombyx mori* L.

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Abstract

An approach has been made to understand the expression of the specific and nonspecific esterase polymorphism in whole body tissue of the silkworm breeds namely, CSR2, CSR4, Pure Mysore and C.nichi. utilizing a polyacrylamide gel stained with α- and β-naphthylacetate as nonspecific hydrolyzing substrate. The genetic diversity among silkworm breeds existed in relation to isozyme of nine esterases, but most of the esterase profiles, such as, Est-4, 5, 6, 7, 8, and 9 found to be noticed as nonspecific utilizing two substrates. The Est-1 is confirmed to be a specific α -esterase in the silkworm as it appears with α -naphthylacetate in CSR2 and CSR4 bivoltine breeds. The Est-2 observed to be nonspecific for all selected breeds except C.nichi, whereas, Est-3 was nonspecific for bivoltine breeds but specific for mutivoltine races. The expression of the isozymes Est-5 and Est-9 are revealed as breed-specific esterases as represented their profiles only in Pure Mysore and CSR4 breed respectively. The outcome of the study interprets the origin of breeds substantiated the significant role of isoenzyme marker for analysis of gene variation between the species in relation to expression of the voltinism in geographically isolated silkworms.

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Introduction

Silkworm genotypes are broadly classified into productive high yielding strains completes two generations per year are called bivoltine (egg diapause) breeds adapted to temperate conditions, however low yielding strains complete five to six generation per year called polyvoltine (egg non-diapauses) silkworm breeds are adapted to tropical climatic conditions. High yielding strains have higher cocoon weight, cocoon shell weight, shell ratio and better in their qualities of the silk in comparison to low yielding strains but are highly susceptible to occurrence of diseases (Shivakumar *et. al.*, 2011). However, from the productivity point of view, bivoltine are preferred. Since the bivoltine are prone to abiotic and biotic stresses, attempts are being made to develop silkworm hybrids with productivity traits of bivoltine breeds and temperature tolerance were observed in polyvoltines. In order to achieve the goal, knowledge of the genetic makeup of the silkworm strains is necessary (Awasthi *et al.*, 2008). Therefore studying silkworm phylogenetic diversity is substantial for selection of functional parent and specific fingerprints. Application of isoenzymes and other molecular markers help to estimate genetic diversity much more accurately than morphological traits. Electrophoresis identifies variation (alleles) at loci that codes for expression of enzyme activity, in addition Chandrakanth *et. al.*, (2014) has revealed the analysis of genetic variability in some silkworm strains of *B. mori* L. through isoenzyme markers; esterase and acid phosphatase.

Insect esterases are grouped according to their reactions to different substrates. In silkworm, midgut esterases were classified into six different types and had a relation between race and esterase type (Yoshitake, 1963). Eguchi and Sugimoto (1964) reported five different esterase bands in the haemolymph of the silkworms with an orientation of gradual changes in the enzymatic activity of each esterase band during ontogeny. Eguchi *et al.* (1965) also described the types and inheritance of haemolymph esterase in the silkworm. They stated that four fundamental types of esterase were controlled by co-dominant alleles BesA, BesB, BesC and BesO (Blood Esterase). About 70% of Japanese, Chinese and European races belong to A type and 20% to O type, while B type is only found in the Chinese race. Chattopadhyay *et al.*, (2001) documented the esterase isozyme polymorphism for digestive juice and haemolymph of the tropical multivoltine silkworm, *Bombyx mori* L., breed CB5 and its syngenic lines using α- and β-naphthylacetate separately as nonspecific substrates, polymorphism existed in the isozyme pattern of α-esterase with two or three bands in digestive juice and three to five bands in haemolymph. Beside polymorphism of nonspecific esterases from pupal haemolymph was analyzed, as well as of phosphorglucomutase from different organs of larvae, pupae and imago, from eight introduced breeds from Bulgaria described by Staykova (2008).

We found little attention paid to voltinism expression of esterase isozyme during the larval period in *B. mori*. Hence present study aimed to investigate the existing polymorphism of specific and nonspecific esterase at two esterase loci, to highlight the heterogeneity and activities of α- and β-esterase, to maintain the variability within and among population for conservation of genetic resources, and detect the relationship among different breeds and their votinism. Further, the intention was to identify genetical marker(s), for quantitative trait to be informative for improvement of them in a marker assisted breeding programme, and distinguishing their geographical origin by their isozyme profile pattern for enhancing inherent characters to produce better silk quality. The breeds reared in Mysore were not yet analyzed for studying polymorphic esterase genotypes. None of the authors mentioned the presence of specific α- and β-esterase bands in the entire tissue of selected silkworms in relation to the breed-specific voltinism by using α- and β-naphthylacetate as a substrate, and it would be suitable to study the regulation of diapause in insect related genetic expression.

Materials and methods

Collection of samples

Four disease-free layings of two bivoltine strains of the silkworm *B. mori* namely, CSR2, CSR_4, and two multivoltines namely,Pure Mysore and C.nichi, showing different in morphological traits originating from India, China and Japan (Table -1) were maintained at Germplasm Bank, Department of Studies in Sericulture Science. Pure Mysore (Karnataka , India) is unique in that it takes more than 28 days to complete its larval life as compared to the 20-22 days observed for almost all of the multivoltine strains. C.nichi, of the Chinese origin is said to be of the bivoltine type when it was brought to India from Japan almost 80 years ago, and has become more of a multivoltine type with associated characters, as a result of continuous breeding under tropical conditions (Reddy *et al*., 1999), but the bivoltine strains attain higher body weight, secretes longer silk fiber of superior quality, and show susceptibility to different pathogens and to high levels of heat, humidity, and inadequate sanitary conditions during silkworm rearing therefore there is not much information is available on genetic diversity and expression of voltinism in the bivoltine silkworm breeds.

The entire larval body of each breeds were selected randomly at age of Vth instar fifth day, was considered for crushing and collected into a chilled 1.5 ml microfuge tube then centrifuged at 10000 rpm for 10 minutes in a cooling centrifuge at 4°C. The supernatant was placed in EDTA-coated tubes and stored at –20°C for future use.

Polyacrylamide gel electrophoresis (PAGE)

Fifteen µl of each sample was subjected to electrophoresis under natural conditions on a 8.25% polyacrylamide gel following the procedure as described by Ayala *et al.,* (1972). The PAGE was carried out initial voltage of 50 and followed by 100 V for 3 h until the tracing dye reached the bottom of the gel. Gels were stained for α- and β-esterase activities separately. Gels were rinsed rapidly in ice-cold distilled water and placed separately in a tray containing 1 ml of 2% α- or β-naphthylacetate in acetone, 50 mg of fast Blue BB salt, and 100 ml of 0.1 M sodium phosphate buffer, pH 6.5. Gels were incubated under dark conditions at 30°C for 8 h. Controls were similarly incubated but in the absence of α- and β-naphthylacetate.

Controls were similarly incubated but in the absence of α- and β-naphthylacetate. Gels were documented in the System GDS-7600-UVP Ltd, UK transilluminator and separate photographs were also taken. The relative mobility (*R*f value) was calculated for each polypeptide from the formula: *R*f =distance of protein migration /distance of dye migration (Shi and Jackowski, 1998).

Results

The genetic variability of the silkworm breeds has represented with isozyme profile of the nine esterases, which most of them, Est-4, 5, 6, 7, 8, and 9 found to be noticed as nonspecific as they exhibited utilizing two different substrates separately.

Table 1. Lineages of races and their origin.

Lineage	Origin	Voltinism
Line-1 (PM)	Indo-China	Multivoltine
Line-2 (C.nichi)	Japanese	Multivoltine
Line-3 (CSR_2)	CSR & TI Mysore, India	Bivoltine
Line-4 (CSR4)	CSR & TI Mysore, India	Bivoltine

The α -Est-1 with *Rf* value of 0.24 confirmed to be a specific esterase in silkworm *B. mori* as it only appears with help of α -naphthylacetate in CSR2 and CSR4 strains belong to India, as well as a voltinism-specific for selected bivoltine breeds. The difference in *Rf* value (Table 2) for each band suggested the existence of isozyme polymorphism among the four lines. The line Pure Mysore (PM) displayed almost similar banding pattern with another multivotine breed, whereas, PM consisting prominent presence of

α-Est-5 with *Rf* value of 0.50, however was not appeared in C.nichi (Figure 1, lane 2). Every larvae display bands α-Est-2, 3, 4, 6 and 7 stained by α - naphthylacetate (Figure 1). Est-8 with *Rf* value of 0.72 observed to be a nonspecific esterase but voltinism-specific because it was absent in multivoltine breeds whereas present in bivoltine breeds with use of both substrates separately.

Table 2. The relative mobility (*R*f) value of each α- esterase band of tissue in of *B. mori* breeds. Lane 1, Pure Mysore; lane 2, C.nichi (multivoltine); lane 3, CSR2; lane 4, CSR4 (bivoltine); using α-naphthylacetate separately as a substrate**.**

α-Esterase bands	Lane 1	Lane 2	Lane 3	Lane 4
EST-1	-	-	0.24	0.24
EST-2	0.32	0.32	0.32	0.32
EST-3	0.40	0.40	0.40	0.40
EST-4	0.45	0.45	0.45	0.45
EST-5	0.50	-	-	-
EST-6	0.56	0.56	0.56	0.56
EST-7	0.62	0.62	0.62	0.62
EST-8	-	-	0.72	0.72
EST-9	-	-	-	0.78

β-esterase isozyme banding pattern in entire tissue from larvae of the same lines depicted eight bands as β- Est-2, 3, 4, 5, 6, 7, 8 and 9 (Figure 2, lanes 1–4) and the polymorphism existed among them as *Rf* values has shown for each esterase band of each line (Table 3).

Table 3. The relative mobility (*R*f) value of each β- esterase band of tissue in *B. mori* breeds. Lane 1, Pure Mysore; lane 2, C.nichi (multivoltine); lane 3, CSR2; lane 4, CSR4 (bivoltine); using β-naphthylacetate separately as a substrate.

β-Esterase Bands	Lane 1	Lane 2	Lane 3	Lane 4
EST-1	-	-	-	-
EST-2	0.32	-	0.32	0.32
EST-3	-	-	0.40	0.40
EST-4	0.45	0.45	0.45	0.45
EST-5	0.50	-	-	-
EST-6	0.56	0.56	0.56	0.56
EST-7	0.62	0.62	0.62	0.62
EST-8	-	-	0.72	0.72
EST-9	-	-	-	0.78

The Est-2 with *Rf* values 0.32 observed to be nonspecific for all breeds except C.nichi, origin of Japan as it did not exhibit in lane 2 with use of β-naphthylacetate, also Est-3 with *Rf* value of 0.40 was nonspecific for bivoltine but specific for multivotine breed. All the larvae exhibited alleles β -Est-4, 6 and 7, but there was lack of Est-3 allele in multivoltine strains with help of this substrate (see figure 2).

The expression of α-esterases alleles in selected *B. mori* lineages, and their *Rf* values with help of α naphthylacetate and β –esterase by β - naphthylacetate has been depicted (see figure 3 and 4). The α-esterase isozyme pattern in C.nichi (multivoltine) of entire tissue showed the maximum heterogeneity for three to five bands, which were designated as α - Est-2, 3, 4, 6, and 7 compare with other silkworm breeds.

The esterase bands showed intensity variation among the silkworm strains with different voltinism and origin, and its understanding showed the Est-4 was deeply stained in bivoltine breeds and in case of multivoltine, C.nichi was faintly stained compare with Pure Mysore in same Est-4, as well as Est-6 and 7 which were stained more in bivoltine breeds with help of β- naphthylacetate (Table 4).

Table 4. Electrophorestic banding pattern showing the intensity variation of esterase isozymes in entire tissue of *B. mori* breeds using β - napthylacetate as substrate. Lane 1, Pure Mysore; lane 2, C.nichi (multivoltine); lane 3, CSR2; lane 4, CSR4 (bivoltine). '+', '++', '+++', '-' denotes faintly, medium and deeply stained and absent in substrate respectively.

Esterase β-Bands	Lane 1	Lane 2	Lane 3	Lane 4
EST-2	+	+	+	+
EST-3	-	-	++	++
EST-4	++	+	+++	+++
EST-5	+++	-	-	-
EST-6	++	++	+++	+++
EST-7	++	++	+++	+++
EST-8	-	-	+	+
EST-9	-	-	-	+

Discussion

As the global scenario in present trend is now moving towards utilization of molecular markers playing considerable characteristics for elucidation of evolutionary origins and relationships in all living organisms the analysis of phylogenetic diversity in the silkworm, *B. mori* can prove this fact.

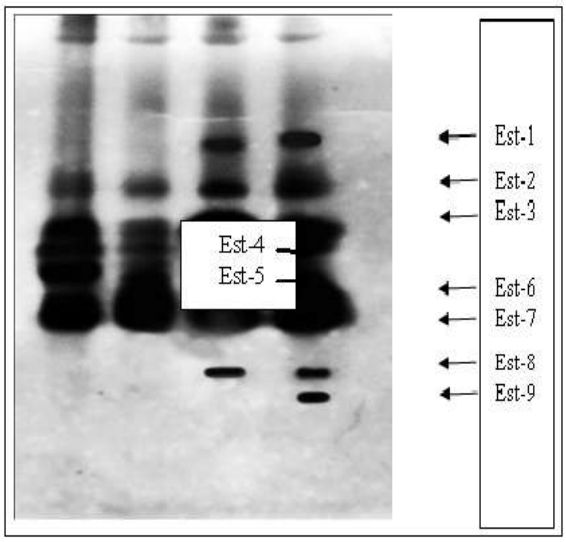

Fig. 1. Vertical 8.25% PAGE of α -esterase isozyme pattern of tissue of *B. mori* lines. Lane 1, Pure Mysore; lane 2, C.nichi; lane 3, CSR2; lane 4, CSR4; the bands EST-4, 5, 6, 7, 8, and 9 were nonspecific and Est-1was specific in bivoltine lanes whereas Est-3 was specific using 2% α -naphthyalacetate as a substrate in multivoltine lanes. Specific and nonspecific α -esterase isozyme bands were identified by the absence or presence in the PAGE of a β - esterase isozyme pattern of the same samples using β - naphthylacetate as a substrate.

The recent investigations by different molecular approaches such as and DNA and isoenzymes markers, their application indicate the significance of the conservation in silkworm races/species. The domesticated silkworm, *B. mori*, is an herbivorous insect and a lepidopteran model organism. Chinese and Japanese scientists made great efforts to accomplish the sequencing project of the whole silkworm genome, which offers researchers an opportunity to identify peptides using proteomic method (Mita *et al.*, 2004).

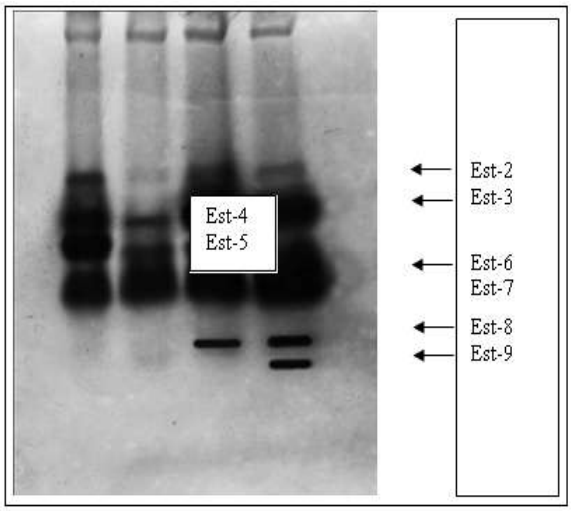

Fig. 2. Vertical 8.25% PAGE of β esterase isozyme pattern of tissue of *B. mori* lines. Lane 1, Pure Mysore; lane 2, C.nichi; lane 3, CSR2; lane 4, CSR4; the esterase bands Est-4, 5, 6, 7, 8, and 9 were nonspecific using 2% β -naphthylacetete as substrate. Nonspecific β -esterase isozyme bands were identified by the presence in the PAGE of α -esterase isozyme pattern of the same samples using α -naphthylacetate as substrate.

Besides the improvement the isozymes may

contribute to the understanding the expression of functional genes, in different tissues or organs and in different phases of the development in *B. mori*. Genotypes analysis of different strains can be used in future studies of genetic improvement to develop superior hybrids for silk production (Ronqui *et al.*, 2012). Multi-molecular forms of esterase isozyme patterns in midgut tissue and haemolymph of silkworm have been studied by various workers (Yoshitake, 1963; Eguchi and Sugimoto, 1964; Eguchi *et al.*, 1965; Stoikova *et al.,* 1998).

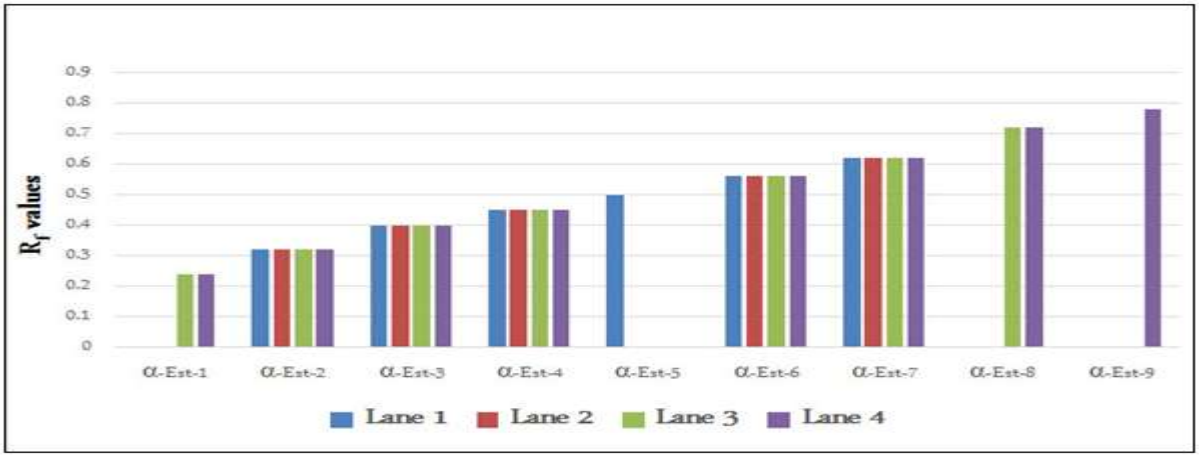

Fig. 3. α-esterase activity of nine alleles in individual larvae with help of α -naphthylacetate (Relative mobility of Lane 1, Pure Mysore; lane 2, C.nichi; lane 3, CSR2; lane 4, CSR4).

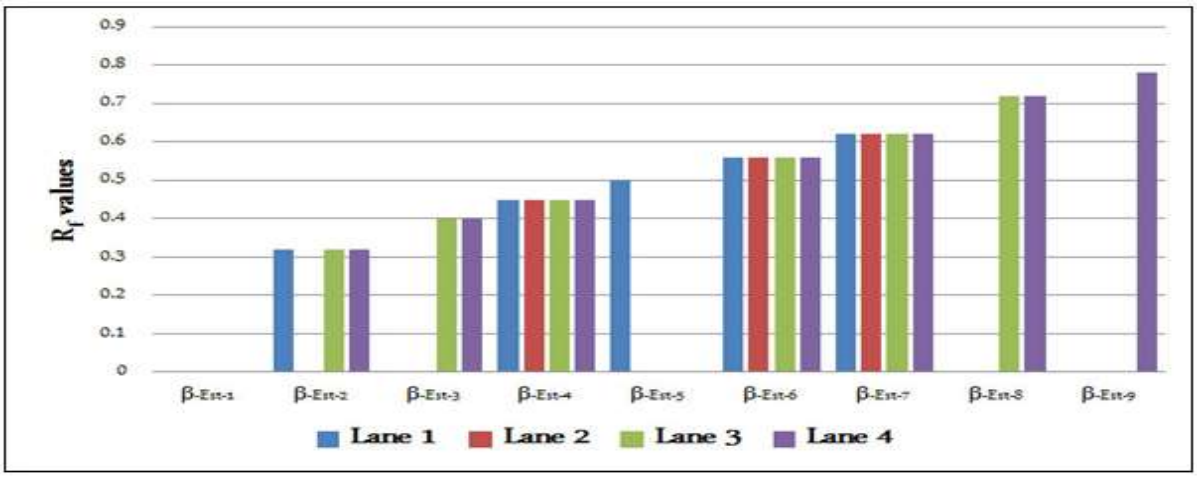

Fig. 4. β -esterase activity of nine alleles in individual larvae with help of β -naphthylacetate (Relative mobility of Lane 1, Pure Mysore; lane 2, C.nichi; lane 3, CSR2; lane 4, CSR4).

Further Chattopadhyay *et al.*, (2001) revealed the esterase isozyme polymorphism of specific and nonspecific esterase, syngenic lines development and natural occurrence of a thermostable esterase in the tropical silkworm, *Bombyx mori* using α- and β-naphthylacetate as substrate where the races or breeds originated either from temperate or tropical regions. Their report documented the presence of specific β-esterase bands (Est-1, 2 and 3) in digestive juice and α-esterase bands (Est-1, 4 and 5) in haemolymph using the same substrates separately, the other bands in both samples (digestive juice and haemolymph) were nonspecific. Therefore, their results suggested that α- and β-naphthylacetate act as both nonspecific and specific substrate. Heterozygosity and alleles frequencies at polymorphic specific and non-specific esterases in pupal haemolymph with polylocus control have been genetically determined in eight introduced breeds of the silkworm in Bulgaria by Staykova (2008).

Our study infers the isozyme esterase from the entire body tissue is better suited for investigating of inter and intra-breed polymorphism and establishing the specific and nonspecific esterase in relation to voltinism in mulberry silkworm as the profile pattern confirmed α -Est-1as specific esterase and the nonspecific Est-8 was appeared only in bivoltine breeds whereas α–Est-3 was exhibited in multivoltine. Besides this research has ascertained the breed specificity in selected *B. mori* indicating their ecological-geographic origin like the Est-9 as it presented merely in lane 4 (Figure 1 and 2) and made it a unique esterase for CSR4 even though it is from the same origin and voltinism with CSR2. Also Pure Mysore possessed a breed-specific Est-5 that maybe due to its Indo-Chinese particular origin and discriminated it even from the other multivoltine, C. nichi, origin of Japan which represented specific α -Est-2 but not β- Est-2. The results of this study provide new information concerning the enzyme polymorphism in different strains in relation to exhibit the manifestation of voltinism in mulberry silkworm.

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